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## Regulation of heat shock protein 70-1 expression by androgen receptor and its signaling in human prostate cancer cells

Shan Lu<sup>1</sup>, Zongqin Tan<sup>1</sup>, Matt Wortman<sup>2</sup>, Shan Lu<sup>3</sup>, and Zhongyun Dong<sup>1</sup>

<sup>1</sup>Department of Internal Medicine, The University of Cincinnati College of Medicine, Cincinnati, OH 45267, USA

<sup>2</sup>Department of Cancer and Cell Biology, The University of Cincinnati College of Medicine, Cincinnati, OH 45267, USA

<sup>3</sup>Department of Pathology and Laboratory Medicine, The University of Cincinnati College of Medicine, Cincinnati, OH 45267, USA

### Abstract

Heat shock protein (hsp) 70-1 (hsp70-1) is overexpressed in human prostate cancer cells and may play important roles in prostate cancer resistance to conventional therapies. The purpose of this study was to investigate whether androgen receptor (AR) and its signaling regulate hsp70-1 expression. Several lines of AR-positive (LNCaP, LAPC-4, and 22Rv1) and -negative (PC-3, DU145, WPE1-NB14 and WPE1-NB-26) human prostatic cells were used in the study. Dihydrotestosterone (DHT) enhanced hsp70-1 expression in LNCaP cells. Expression of hsp70-1 in LNCaP cells was downregulated by the anti-androgens bicalutamide (Bic), and flutamide (Flut), and a newly identified AR signaling antagonist DL3. The downregulation of hsp70-1 by DL3 was also observed in LAPC-4 and 22Rv1 cells, but not in the four lines of AR-negative cells examined. Expression of hsp70-1 was also reduced by DL3 in PC-3 cells engineered with AR. On the other hand, knocking down AR in LNCaP cells by siRNA moderately reduced hsp70-1 level and abolished effects of DL3 on hsp70-1 expression. DL3 reduced hsp70-1 mRNA synthesis in cells and its *in vitro* gene transcription but did not significantly alter the stabilities of hsp70-1 mRNA and protein. Chromatin-immunoprecipitation (ChIP) assay showed that AR bound to the promoter region of *HSPA1B* gene, which was reduced in cells treated with DL3 or Bic. These data suggest that AR and its signaling regulate hsp70-1 expression in prostate cancer cells and that *HSPA1B* could be an AR target gene.

### Keywords

androgen receptor; heat shock protein 70-1; antagonist; prostate cancer

### Introduction

Heat shock proteins (hsp) are encoded by several gene families and play essential roles in cell survival, tumorigenesis, and tumor progression (1,2). The hsp70 family proteins, named according to their approximate relative molecular mass, contain at least 8 members that are almost ubiquitously expressed (3,4). The two major hsp70 proteins are hsp70-1a and hsp70-1b with amino acids being >99% identical and are encoded by *HSPA1A* and *HSPA1B* genes, respectively (3,4). They were initially found in cells under stress (3,4). There are also several

hsp70 homologues that are constitutively expressed and are termed as heat shock ‘cognate’ proteins (Hscs). Hsc70 and hsp70 are highly homologous and have identical biochemical properties (5) and similar domain structures, including an N-terminal ATPase domain, a peptide-binding domain with the EEVD motif mediating hsp70 interaction with tetratricopeptide repeat (TPR)-containing client protein, and a C-terminus (5). Along with hsp90 and several co-chaperones, hsp70 plays critical roles in maintaining protein integrity and synthesis, such as folding and assembling AR to a high-affinity ligand binding conformation (1,6-8).

Hsp70-1 proteins are overexpressed in many types of tumor and this overexpression of hsp70-1 is often correlated with tumor malignancy, and progression (1,5,9), poor prognosis and presence of metastasis (1,5,9,10). Overexpression of hsp70-1 induces cell transformation (5, 11,12). These effects of hsp70-1, possibly through downregulation of mammalian sterile 20-like kinase 1 (13), are due partially to its protection of cells from apoptosis and/or necrosis induced by chemotherapeutic agents and other stress stimuli (14-16). Indeed, depletion of hsp70-1 expression was shown to be sufficient to trigger apoptosis in several types of cells (17). Hsp70-1 expression is elevated in prostate cancer (9). Although depletion of hsp70-1 using siRNA and antisense RNA did not alter the cell viability, it did enhance sensitivity of prostate cancer cells to a variety of anticancer agents (9), suggesting that downregulation of hsp70-1 could be an effective approach for combination therapies to overcome the natural resistance of prostate cancer to chemotherapies.

Expression of hsp70-1 is mainly regulated by heat shock factor 1 (HSF1) that binds to heat shock transcription control element. Interestingly, it has been shown that androgen, through activation of AR, induces hsp70-1 expression in ventricular myocytes (18). Moreover, overexpression of AR and other steroid receptors has been shown to activate HSF1 in COS-1 fibroblasts (19). These observations clearly suggest that AR signaling regulates hsp70-1 expression.

The purpose of this study was to determine potential roles of AR signaling in regulation of hsp70-1 expression in prostate cancer cells. We show that DHT enhanced hsp70-1 mRNA expression, which was attenuated by a newly identified AR signaling antagonist DL3 (20). Expression of hsp70-1 protein was also downregulated by DL3 and the non-steroid antiandrogens Bic and Flut. DL3-induced downregulation of hsp70-1 was more moderate in AR-negative cells and was enhanced when AR-negative cells are forced to overexpress AR. Treatment with DL3 reduced hsp70-1 mRNA gene transcription but had marginal effects on hsp70-1 mRNA stability. Finally, AR bound to promoter region of the *HSPA1B* gene, which was attenuated by DL3 and, to a lower extent, by Bic.

## Materials and methods

### Tumor cells and culture

LNCaP (21,22), 22Rv1 (22,23), WPE1-NB14 and WPE1-NB26, PC-3, and DU-145 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). LNCaP and 22Rv1 cells were maintained in RPMI-1640 supplemented with 10% FBS. WPE1-NB14 and WPE1-NB-26 cells were cultured in the keratinocyte serum-free medium (K-SFM) supplemented with bovine pituitary extract (50  $\mu$ g/ml) and epidermal growth factor (5 ng/ml). PC-3 and DU-145 cells were cultured in MEM supplemented with 5% FBS. PC-3AR cells, derived from PC-3 cells by stable transfection of wild-type AR, were generously provided by Dr Zhengxin Wang (Department of Cancer Biology, The University of Texas M.D. Anderson Cancer Center, Houston, TX). LAPC-4 cells (22,24) were generously provided by Dr Karen Knudsen (Kimmel Cancer Center, Thomas Jefferson Medical College, Philadelphia, PA).

LAPC-4 cells were maintained in IMDM medium supplemented with 10% FBS and 10 nM of DHT.

### Immunoblot analysis

Cells ( $2 \times 10^6$ /60-mm dish) were plated and treated after an overnight incubation as desired. The cells were washed in cold PBS/5 mM EDTA, scraped into a lysis buffer, and analyzed by Western blotting (25) using antibodies against PSA (DakoCytomation, Glostrup, Denmark), AR (Epitomics, Burlingame, CA), hsp70-1 (Cell Signaling Technology, Inc., Danvers, MA), hsp90 (Santa Cruz Biotechnology, Santa Cruz, CA), and  $\beta$ -actin (Santa Cruz). The immunoreactive signals were revealed using an ECL Western blotting detection system (Michigan Diagnostic LLC, Troy, MI) and visualized in a Kodak IS4000MM Image Station (Eastman Kodak Co., Rochester, NY).

### qRT-PCR

Cells were seeded at  $2 \times 10^6$  cells/60-mm dish. After an overnight incubation, the cells were treated in fresh medium for various times, washed, and total RNA was extracted using TRIzol reagent. Expressions of AR, PSA, and hsp70-1 were analyzed by qRT-PCR (26) using the following primer pairs: AR, 5'-GGTGAGCAGAGTGCCCTATC-3'/5'-GGCAGTCTCCAAACGCATGTC-3'; PSA, 5'-GACCACCTGCTACGCCTCA-3'/5'-AACTTGCGCACACACGTCATT-3'; hsp70-1, 5'-CGAGGCGGACAAGAAGAA-3'/5'-GATGGGGTTACACACCTGCTC-3' and  $\beta$ -actin, 5'-CGGCACCACCATGTACCCT-3'/5'-ACACGGAGTACTTGCGCTCAG-3', respectively. The cycle threshold values were used to calculate the normalized gene expression against  $\beta$ -actin using the Q-Gene software.

### Nuclear run-on assay

A qRT-PCR-based assay was performed (26). Briefly, cells were rinsed with cold PBS and scraped in lysis buffer on ice, followed by addition of the same volume of 0.6 M sucrose buffer. Cell nucleus pellets, obtained by centrifugation at  $500 \times g$  for 10 min, were incubated for 30 min at  $30^\circ\text{C}$  in 500  $\mu\text{l}$  of transcription reaction buffer (pH 7.9) containing 50 mM Tris, 5 mM  $\text{MgCl}_2$ , 0.1 mM EDTA, and 0.5 mM NTP. RNA was extracted and followed by DNase treatment and qRT-PCR. Samples without transcription reaction were used as controls.

### Transient transfection

Cells ( $10^6$ /60-mm dish) were transiently transfected with an expression vector encoding human hsp70-1 (pCMV6-XL5, OriGene, Rockville, MD), AR-specific siRNA (On-Target plus Smartpool), or a scrambled control siRNA (Dharmacon, Inc., Chicago, IL) using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). Twenty-four to 48 h later, expressions of AR and hsp70-1 were determined by immunoblotting.

### Chromatin immunoprecipitation (ChIP) assay

Cells were plated into 150 mm dish ( $1.5 \times 10^7$ /plate). After an overnight incubation, the cells were treated for 4 h with 20  $\mu\text{M}$  of DL3 or Bic, fixed in 1% formaldehyde for 10 min at room temperature, rinsed with cold PBS, and incubated in PBS containing 125 mM glycine for 5 min. The cells were then scrapped into PBS supplemented with 5 mM PMSF and pelleted. The ChIP assay was carried out using a kit from Upstate Biotechnology (Lake Placid, USA). Briefly, cells were lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) with protease inhibitors and sonicated using Sonicator S-4000 (Misonix, NY) to shear DNA. AR-chromatin-transcription factor complex was precipitated with an antibody to AR (C19, Santa Cruz) or normal rabbit IgG (control). With 1:150 dilution of total DNA as a positive control (total input), DNA obtained from the immunoprecipitation was analyzed by using qPCR. The primer pair encoding the promoter region of human *HSPA1B* gene (5'-

GGTCCGCTTCGTCTTTTCG-3'/5'-CTCTGTGGGCTC CGCTCT-3') was published by others (27) (accession no. NM005346). Data from ChIP assay were presented as the ratio between precipitated DNA over total input. A portion of the ChIP samples was analyzed by immunoblotting to evaluate AR levels. In a parallel setting, total cell lysates were precipitated with the same AR antibody to evaluate the total cellular AR level in each treatment group.

### Statistical analysis

All experiments were performed at least twice. Data shown are the mean  $\pm$  SE. Differences between means were compared using the two-tailed Student's t-test and were considered significantly different at the level of  $p < 0.05$ .

## Results

### Effects of DHT and DL3 on AR, PSA, and hsp70-1 expression

Expression of heat shock proteins, including hsp70-1 and hsp90, are elevated in human prostate cancer cells. Regulation of their expression is, however, not well studied. We determined whether hsp70-1 and hsp90 expression in LNCaP cells was regulated by AR signaling in cells treated with DHT and DL3, a novel AR signaling antagonist identified in our previous study (20). LNCaP cells were incubated for 48 h in medium supplemented with 10% SFBS, then treated for 24 h in fresh medium supplemented with the SFBS with increasing concentrations of DHT in the absence or presence of 20  $\mu$ M of DL3. The cellular protein was analyzed by immunoblotting. As shown in Fig. 1A, DHT enhanced AR and PSA protein levels in a dose-dependent manner. These effects of DHT were abrogated by the presence of DL3. In contrast to the expression of AR and PSA, expression of neither hsp70-1 nor hsp90 was significantly altered by DHT (Fig. 1A). Treatment with DL3 reduced hsp70-1 expression, regardless of the absence or presence of DHT (Fig. 1A). In contrast, DL3 decreased hsp90 expression only in the presence of 10 nM DHT, a concentration of androgen much higher than those physiologically achievable levels. The qRT-PCR analysis revealed that treatment of DHT (1 nM) did not significantly alter AR mRNA expression but enhanced the expression of both PSA and hsp70-1 mRNA (Fig. 1B). The DHT-enhanced expression of both PSA and hsp70-1 mRNA was attenuated by the presence of DL3 (Fig. 1B).

### Downregulation of hsp70-1 expression by DL3 and the anti-androgens

To further explore potential roles of AR signaling in regulation of hsp70-1 and hsp90 expression, the time course and dose-dependent effects of DL3 were determined. LNCaP cells in complete medium were plated and treated with 20  $\mu$ M of DL3. At various times after the treatment, the cellular protein was harvested and analyzed by immunoblotting. As expected, DL3 downregulated expressions of both AR and PSA in a time-dependent manner. Expression of AR and PSA were reduced by 33 and 62%, respectively, at 8 h after the treatment and further decreased thereafter. Expression of PSA was diminished in cells treated with DL3 for 24 h (Fig. 2A). These observations confirmed findings reported in our previous study (20). Expression of hsp70-1 was reduced by ~50%. This occurred, however, only in the cells treated by DL3 for 24 and 48 h (Fig. 2A). In contrast, treatment of DL3 did not significantly alter hsp90 expression at any times studied (Fig. 2A). The dose-response analysis showed that expression of PSA was more sensitive to the inhibitory effects of DL3 in comparison with those of AR and hsp70-1. Whereas a significant reduction of PSA was observed in cells treated by 5  $\mu$ M of DL3 (by 70%), down-regulation of both AR and hsp70-1 was observed in cells exposed to DL3 at concentrations  $> 10 \mu$ M (Fig. 2B). Consistent with the time course study above, it appeared that DL3-induced downregulation of AR protein expression was relatively more significant than that of hsp70-1 (Fig. 1B). To validate that downregulation of hsp70-1 by DL3 was due to an attenuation of AR signaling, the effects of Bic and Flut, the two non-steroid antiandrogens most commonly used in prostate cancer therapies, on hsp70-1 expression

were determined. As shown in Fig. 2C, treatment with both Bic and Flut reduced hsp70-1 expression in LNCaP cells. In agreement with their effects on AR and PSA expression, downregulation of hsp70-1 by these two drugs was relatively more moderate in comparison with that of DL3 (Fig. 2C).

The qRT-PCR analysis of cells cultured for up to 48 h showed that AR mRNA levels were increased in the first 24 h after incubation in fresh medium and moderately reduced during the second 24 h (Fig. 2D). An elevation of both PSA (Fig. 2E) and hsp70-1 (Fig. 2F) mRNA was also observed, which occurred much earlier and peaked at ~8 h after an incubation in fresh medium, followed by a continuous decline to approximately basal level at 48 h (Fig. 2E and F). Treatment with 20  $\mu$ M of DL3 reduced respective mRNA expression of all three genes (Fig. 2D-F). However, a significant reduction of AR mRNA occurred earlier at ~4 h and those of PSA and hsp70-1 mRNA at 8 h after an exposure to DL3. These data concluded that DL3 downregulated AR, PSA, and hsp70-1 at both protein and mRNA levels and its effects were more significant at mRNA levels.

### AR-dependent downregulation of hsp70-1 by DL3

Next, effects of DL3 on hsp70-1 expression were further studied in several other human prostatic cell lines with different properties, including LAPC-4 (cancer cells with wild-type AR), 22Rv1 (cancer cells with mutant AR), two AR negative cancer cell lines (PC-3, DU-145), and two transformed AR-negative prostatic epithelial cell lines (WPE1-NB14 and WPE1-NB26). LAPC-4 cells were routinely maintained in medium supplemented with 10 nM of DHT. To be consistent with DHT concentration used in other experiments in this study, 1 nM of DHT was used in the experiment and appeared to cause a moderate reduction of hsp70 in the cells (Fig. 3A). DL3 downregulated hsp70-1 expression in LAPC-4 cells, which appeared to be more significant in cells treated with DHT (Fig. 3A). 22Rv1 cells were relatively refractory to androgen and expressed a relatively lower level of AR and PSA in comparison with those in LNCaP cells (data not shown). A significant downregulation of hsp70-1 in 22Rv1 cells required higher concentration (20-40  $\mu$ M) of DL3 (Fig. 3B). The treatment with DL3 at concentrations up to 40  $\mu$ M did not significantly alter hsp70-1 expression in all the AR-negative cell lines (PC-3, DU-145, PWE-NB14, and PWE-NB26 cells) (Fig. 3C). The hsp70-1 protein expression was further investigated in PC-3 cells stably engineered with recombinant AR. As shown in Fig. 3D, treatment of DL3 did not alter hsp70-1 level in parental PC-3 cells but it did reduce hsp70-1 protein expression in cells engineered with AR (Fig. 3D). On the other hand, whereas knocking down AR expression in LNCaP cells by using AR-specific siRNA but not a scrambled control siRNA only moderately reduced hsp70-1 expression, it attenuated the inhibitory effects of DL3 (Fig. 3E). Overexpression of hsp70-1 enhanced AR expression (Fig. 3F). This hsp70-1-enhanced AR expression was also reduced when cells were treated with DL3 (Fig. 3F). These data suggested that downregulation of hsp70-1 expression by DL3 in prostate cancer cells relied on the presence of AR and downregulation of hsp70-1 in human prostate cancer cells by DL3 was due possibly to an attenuation of AR expression or AR-mediated signaling.

### Effects of DL3 on AR and hsp70-1 stability and gene transcription

The degradation of both AR and hsp70-1 was shown to be mediated by the ubiquitin-mediated proteasome pathway. We, therefore, investigated whether DL3 accelerated the degradation of these two proteins in proteasome. LNCaP cells were treated with DL3 (20  $\mu$ M) and/or a proteasome inhibitor MG132 (5  $\mu$ g/ml) for 12 h. As shown in Fig. 4A, AR expression was downregulated in cells treated with DL3 (lane 1 vs. lane 2). MG132 treatment alone did not significantly alter AR expression (lane 3 vs. lane 1). It, however, abrogated AR downregulation by DL3 (lane 4 vs. lane 2), suggesting that DL3-induced AR downregulation was due to an acceleration of the proteasome-mediated degradation of AR. Hsp70-1 protein level was

significantly elevated in cells treated with MG132 (lane 3 vs. lane 1). In contrast to AR expression, hsp70-1 protein level was significantly reduced by DL3 regardless of the absence (lane 2 vs. lane 1) or presence (lane 4 vs. lane 3) of MG132. Consistent with the observations in previous experiments, expression of hsp90 was not altered in cells treated with DL3 and/or MG132. These data suggest that DL3 downregulated AR and hsp70-1 by different mechanisms and that it downregulated hsp70-1 expression via mechanisms independent of proteasome-mediated degradation.

Effects of DL3 on AR and hsp70-1 protein stability were further studied in cells treated with protein synthesis inhibitor cycloheximide (CHX). In androgen-starved LNCaP cells, treatment with DL3 further reduced AR level by ~50% (Fig. 4B, lane 2 vs. lane 1). AR level was elevated by the presence of DHT (Fig. 4B, lane 3 vs. lane 1), which was abrogated by DL3 (Fig. 4B, lane 4 vs. lane 3). AR expression was diminished in cells treated with CHX, which was reversed by DHT (Fig. 4B, lane 6 vs. lane 5), due to the enhancement of AR stability by DHT (28,29). This effect of DHT on AR expression was abrogated by DL3 (Fig. 4B, lane 7), suggesting that DL3 promoted AR degradation. In contrast, treatment with DL3 downregulated hsp70-1 by ~40% regardless of the presence or absence of either CHX and/or DHT, suggesting that DL3 did not alter hsp70-1 degradation.

Since hsp70-1 mRNA level was significantly reduced in cells treated with DL3, we determined whether DL3 regulated hsp70-1 mRNA stability. Cells were treated with 5  $\mu$ g/ml of actinomycin D (ActD) to block *de novo* synthesis of new RNA and with DL3 (20  $\mu$ M) for various times. Data in Fig. 4C show that hsp70-1 had a short half-life of about 4 h. The half-life of hsp70-1 mRNA was not significantly altered in cells treated with DL3 (Fig. 4C). On the other hand, the nuclear run-on assay showed that transcription of hsp70-1 mRNA *in vitro* by nuclear extracts from DL3-treated cells was reduced ~60-70% (Fig. 4D), suggesting downregulation of hsp70-1 mRNA and, hence, hsp70-1 protein by DL3 was due mainly to suppression of hsp70-1 gene transcription.

To further explore the potential role of AR in regulating hsp70-1 expression, we first performed sequence analysis of both HSPA1A and HSPA1B gene promoters and identified two consensus sequences of androgen response element (ARE) for each of the genes in the core promoter region spanning ~450 bp including both 5'UTR and 250 bp core promoter. ChIP assay was performed to determine whether AR occupied *HSPA1B* gene promoter region. LNCaP cells were incubated for 4 h in medium supplemented with 10% FBS without or with treatment of 20  $\mu$ M of DL3 or Bic. Subsequently, the cells were fixed. Sheared chromatin was immunoprecipitated with control rabbit IgG (data not shown) or AR-specific antibody. The chromatin precipitate was amplified using a primer pair (27) corresponding to the promoter region of *HSPA1B* gene with total DNA (1:150 dilution) as positive control (total input). As shown in Fig. 4E, AR bound to *HSPA1B* promoter region which was reduced by approximately 60 and 35% in cells treated with DL3 and Bic, respectively. The immunoblot analysis showed that treatment for 4 h with DL3 or Bic did not significantly alter the total cellular AR levels, but reduced the chromatin-associated AR in the ChIP samples (Fig. 4F), suggesting that the treatments reduced the occupation of AR to its target genes.

## Discussion

The hsp70-1 proteins are molecular chaperons critical for AR biosynthesis and function (1, 6-8). Its expression and regulation in prostate cancer cells are, however, not well studied. Data presented in this report show that hsp70-1 is constitutively expressed in all human prostatic epithelial cell lines examined, including AR-positive androgen-dependent LNCaP and LAPC-4 prostate cancer cells, AR-positive androgen-independent 22Rv1 prostate cancer cells, AR-negative androgen-independent PC-3 and DU145 prostate cancer cells, and transformed

prostatic epithelial WPE1-NB14 and WPE1-NB26 cells. Treatment of androgen-dependent LNCaP cells with DHT did not significantly alter hsp70-1 protein level but enhances hsp70-1 gene transcription and mRNA synthesis. Expression of hsp70-1, but not hsp90, is downregulated by AR signaling antagonist DL3 and by anti-androgens Bic and Flut. The downregulation of hsp70-1 expression by DL3 requires the presence of AR in the target cells, regardless of their androgen-dependency status, and is observed in PC-3 cells forced to express AR. These findings strongly suggest that AR and its signaling regulate the expression level of hsp70-1 expression in human prostate cancer cells.

It appears that AR and, hence, AR antagonists regulate hsp70-1 expression in prostate cancer cells mainly at the transcription level. This conclusion is supported by several lines of evidence. First, DHT does not significantly alter hsp70-1 protein level but enhances hsp70-1 mRNA expression, which is blocked by DL3. Second, the blockades of protein synthesis using CHX and protein degradation using MG132 do not reverse or attenuate the inhibitory effects of DL3 on hsp70-1 expression. Third, DL3 treatment does not significantly accelerate hsp70-1 mRNA degradation. Fourth, DL3 significantly reduces the transcription of hsp70-1 gene *in vitro* in the nuclear run-on assay. Finally, AR occupies the promoter region of hsp70-1 gene, which is reduced upon a treatment with DL3 or Bic, and the reduction levels correlate with potencies of the inhibitory effects of DL3 and Bic on hsp70-1 mRNA and protein expression. Together with the fact that hsp70-1 is associated with AR in the cytoplasm and undergoes proteasome-mediated degradation upon dissociation from AR in cells exposing to androgen, findings in this study implicate that AR activation induces hsp70-1 expression. This is possibly a feedback mechanism that maintains a relative constant level of hsp70-1 prostate cancer cells (Fig. 5). In AR-positive cells treated with DL3 or an antiandrogen, however, the AR-induced hsp70-1 expression is attenuated and, hence, leads to a downregulation of hsp70-1. On the other hand, the lack of AR in several AR-negative cell lines renders their refractory to DL3-mediated downregulation of hsp70-1, which is reversed when cells are forced to express AR. Consistent with this notion, the knocking down of AR leads a moderate reduction of hsp70-1 level in the cells and abolishes the downregulation of hsp70-1 by DL3.

Several earlier studies showed that hsp70-1 expression levels vary with the cell cycle and are regulated by c-Myc (30-33). The hsp70-1 expression level is elevated by 65% in G2/M phase of the cell cycle and is lowest in G1 phase among G1, S, and G2/M2 phases of the cell cycle (34). Cultivation of LNCaP cells under androgen-deprived conditions arrests cells at G1 phase of the cell cycle (35). Similarly, treatment with the classical antiandrogens Bic and Flut, and DL3 induce G1 arrest in LNCaP cells (data not shown). Therefore, it is conceivable that reduction of hsp70-1 expression in cells treated by DL3 and the classical antiandrogens is due indirectly to the alteration of cell cycle progression. The observations that reduction of hsp70-1 mRNA, simultaneously with PSA, occurs very early (in 8 h) after treatment with DL3 and that AR binds to *HSPA1B* gene promoter region, however, argue against this notion. Taken together, whereas further studies are needed to establish and elucidate mechanisms by which AR regulates hsp70-1 expression in prostatic cells, data presented in this report do strongly suggest that AR and/or AR signaling may directly regulate hsp70-1 gene transcription.

Previous studies have shown that the bioflavonoid quercetin is a potent inhibitor of hsp70-1 expression. Quercetin down-regulates both constitutive and heat-induced synthesis of hsp70-1, inhibits cell growth, and promotes heat-induced apoptosis in a variety of cell lines, including prostate cancer cells (36-40). Interestingly, quercetin also inhibits AR expression and androgen-induced expression of PSA and other AR target genes (41-43), which is similar to that observed by us with DL3 (20). It is, therefore, of interest to further compare effects of DL3 and quercetin on hsp70-1 expression in both prostate cancer and other types of cells. In addition, it is also of interest in determining whether DL3 could regulate heat-induced hsp70-1 expression in both AR-negative and AR-positive cells. Not only do these studies facilitate the

elucidation of roles of AR signaling in regulating hsp70-1 expression, but they may also demonstrate whether DL3 can serve as a potential heat-sensitizer agent for thermal therapy against prostate cancer.

In conclusion, data presented in this study show that: i) DHT enhances hsp70-1 mRNA expression in human prostate cancer cells, which is attenuated by DL3; ii) DL3, as well as antiandrogens Bic and Flut, downregulates hsp70-1 expression in an AR-dependent fashion and occurs at transcription level; and iii) AR occupies the promoter region of *HSPA1B* gene, which is reduced by DL3 and Bic. These data conclude that AR and its signaling regulate hsp70-1 expression in prostate cancer cells. Given the finding that knockdown expression of hsp70-1 sensitizes prostate cancer cells to chemotherapies as well as other therapeutic modalities (9), downregulation of hsp70-1 using AR signaling antagonist DL3 or an antiandrogen may present an attractive strategy for successful management of advanced prostate cancer through combinational therapies.

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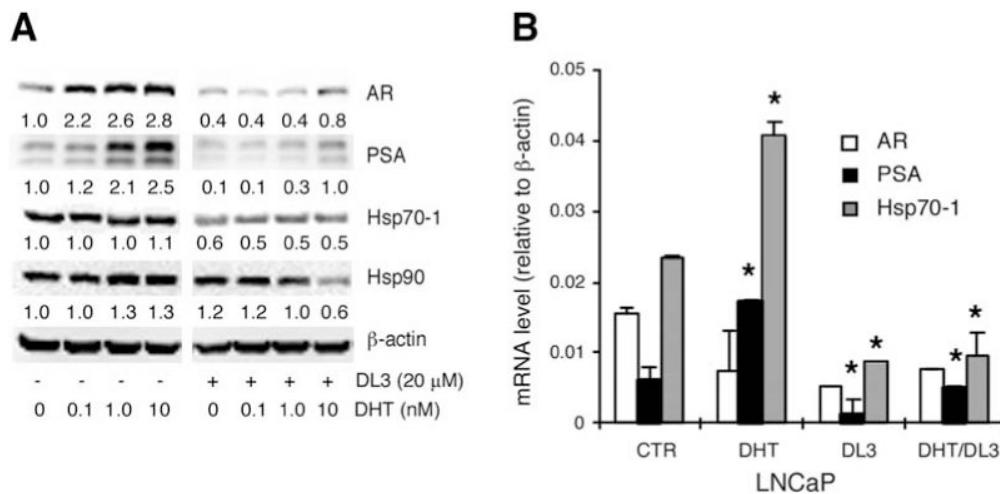


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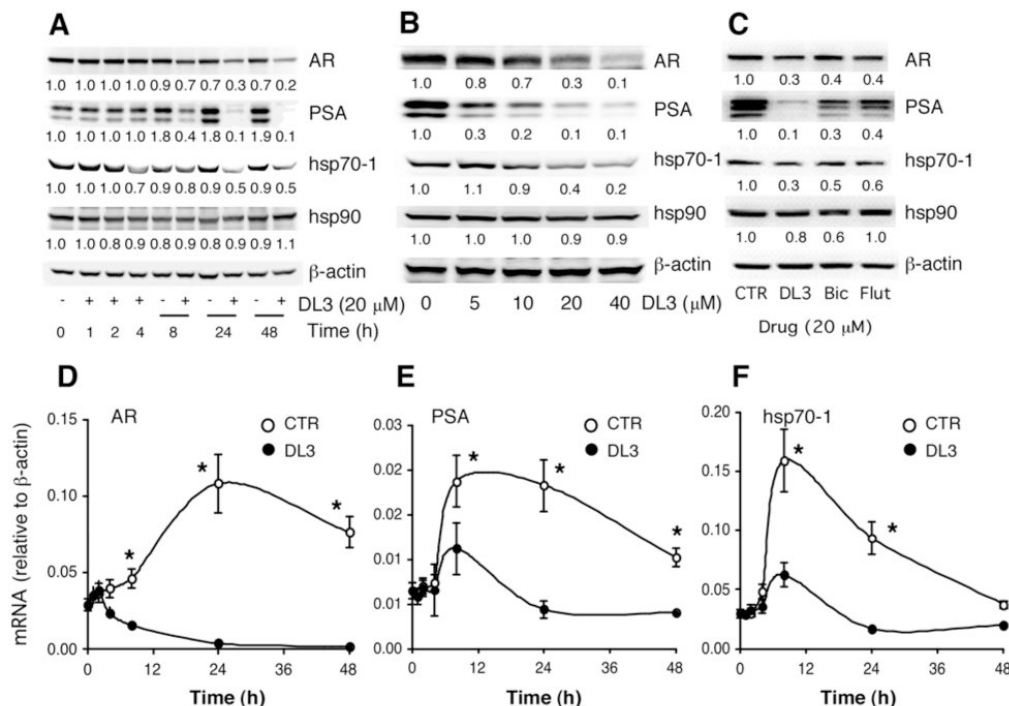
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## Abbreviations

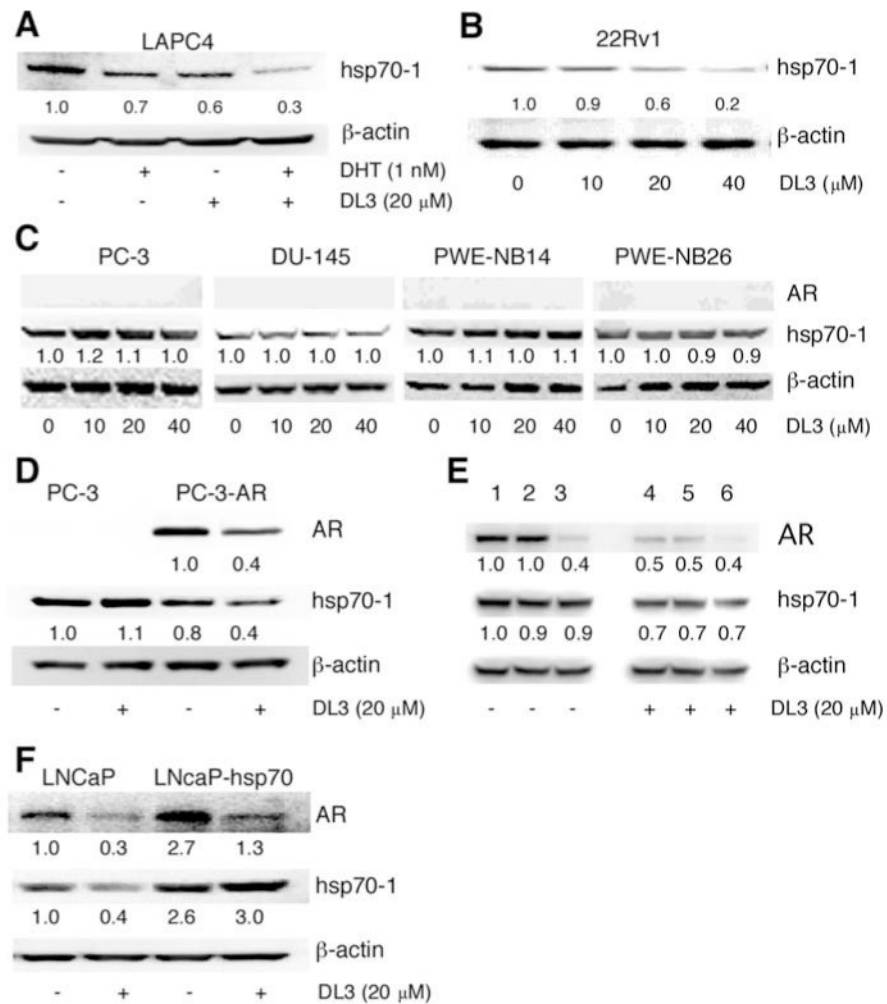
AR	androgen receptor
Bic	bicalutamide
ChIP	chromatin immunoprecipitation
DHT	dihydrotestosterone
DL3	6-amino-2-[2-(4-tert-butyl-p-nenoxy)-ethylsulfanyl]-1H-pyrimidin-4-one
ELISA	enzyme-linked immunoabsorbance assay
FBS	fetal bovine serum
Flut	flutamide
hsp	heat shock protein
HSF1	heat shock factor 1
PSA	prostate-specific antigen
qRT-PCR	quantitative real-time reverse-transcription PCR



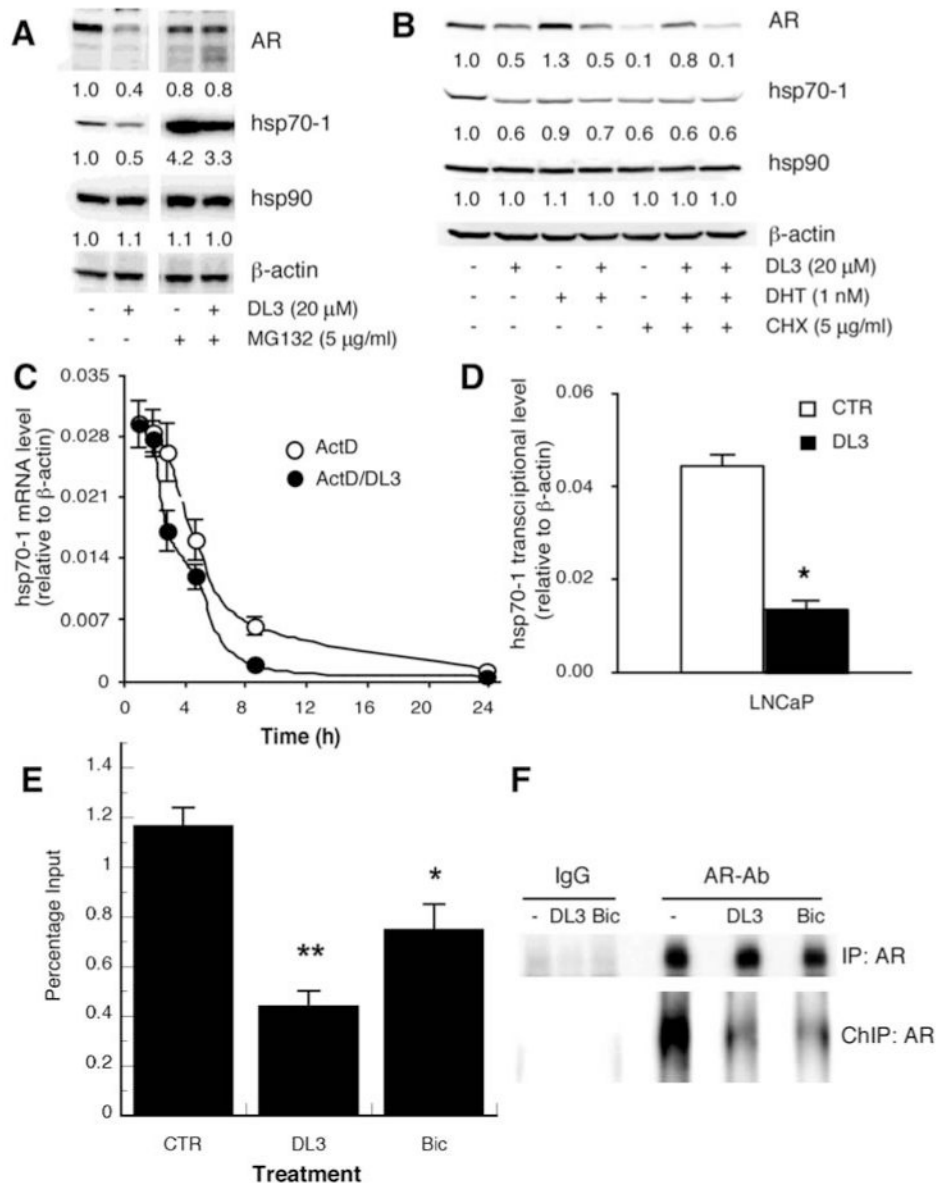
**Figure 1.** Effects of DHT and DL3 on hsp70 and hsp90 expression. (A) LNCaP cells were incubated for 48 h in phenol-free medium supplemented with 10% charcoal-dextran stripped FBS (SFBS), then treated for 24 h with 0-10 nM of DHT in the absence or presence of 20 μM of DL3. Cell lysates were analyzed by immunoblotting with antibodies to AR, PSA, hsp70-1 and hsp90 with β-actin as a loading control. (B) LNCaP cells were treated as described in (A). Total RNA was extracted and expression of AR, PSA, hsp70-1 with β-actin as a loading control was determined by qRT-PCR and presented as ratios to β-actin. \*p<0.05: DHT vs. CTR; DL3 vs. CTR; DHT/DL3 vs. DHT. Data shown were one representative experiment of three.



**Figure 2.** Time- and dose-dependent effects of DL3 on hsp70-1 expression. (A) LNCaP cells were treated for different times in medium supplemented with normal FBS. Cell lysates were analyzed by immunoblotting. (B) Lysates from LNCaP cells treated for 24 h with different concentrations of DL3 were analyzed by immunoblotting. (C) Lysates from LNCaP cells treated for 24 h with 20 μM of DL3, Bic, or Flut were analyzed by immunoblotting. (D and E) LNCaP cells were incubated for various times in medium supplemented with normal FBS or treated with 20 μM of DL3. Total RNA extracted was analyzed by qRT-PCR for the presence of AR (D), PSA (E), or hsp70-1 (F). \*p<0.05: DL3 vs. CTR. Data shown were one representative experiment of three.

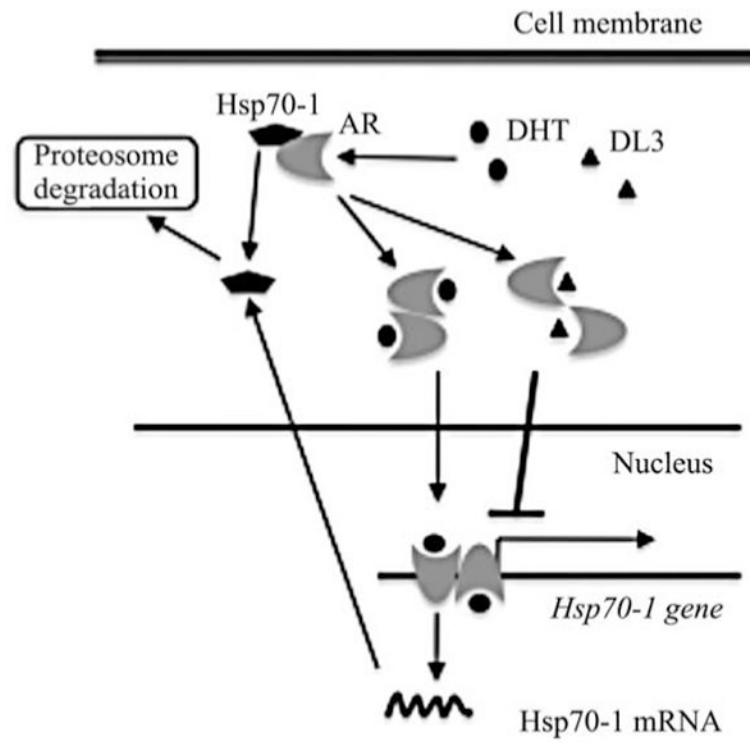
**Figure 3.**

AR-dependent downregulation of hsp70-1 by DL3. (A) LAPC-4 cells were incubated for 48 h in IMDM medium supplemented with 10% SFBS and then treated for 24 h with 1 nM of DHT and/or 20  $\mu$ M of DL3. (B) 22Rv1 cells ( $2 \times 10^6$  cells/well in a 6-well plate) in medium supplemented with 10% FBS were treated for 24 h with different concentrations of DL3. (C) AR-negative PC-3, DU-145, PWE-NB14, and PWE-NB26 cells were treated for 24 h with different concentrations of DL3. (D) PC-3 or PC-3AR, which were stably transfected with wild-type AR were treated for 24 h with 20  $\mu$ M of DL3. (E) LNCaP cells were transfected with AR-specific siRNA (lanes 3 and 6) or a scrambled siRNA (lanes 2 and 5) for 48 h and treated with 20  $\mu$ M DL3 (lanes 4-6) for 24 h. Expression of AR and hsp70-1 in control and treated cells was analyzed by immunoblotting. (F) LNCaP cells were transfected with vector or vector expressing hsp70-1 for 24 h and treated for 24 h with DL3. Lysates from cells described above were analyzed by immunoblotting.



**Figure 4.** Mechanistic study on DL3-induced hsp70-1 downregulation. (A) LNCaP cells in medium supplemented with 10% FBS were treated for 16 h with 20 μM of DL3 and/or 5 μg/ml of MG132. (B) LNCaP cells in medium supplemented with 10% SFBS were treated for 24 h with 20 μM of DL3, 1 nM of DHT, and/or 5 μg/ml of CHX. (C) LNCaP cells were treated for different times with 5 μg/ml of ActD in the absence or presence of 20 μM of DL3. Total RNA was extracted and analyzed by qRT-PCR. (D) LNCaP cells, incubated in medium or treated with 20 μM of DL3, were lysed and mixed with the same volume of 0.6 M isolation sucrose buffer. Cell nucleus pellets obtained were incubated in transcription reaction buffer. Total RNA in the reaction mixture was extracted, and hsp70-1 and β-actin mRNA was assessed by real-time RT-PCR. Samples without *in vitro* transcription reaction were used as controls (not shown). (E) LNCaP cells, treated for 4 h with 20 μM of DL3 or Bic, were fixed in 1% formaldehyde. After washing and incubation in glycine, cells were scrapped into PBS

supplemented with 5 mM PMSF, pelleted, resuspended in SDS lysis buffer, and sonicated to shear DNA. AR-chromatin-transcription factor complex was precipitated with antibodies to AR with IgG from normal rabbit as a negative control. With 1:150 dilution of total DNA as a positive control (total input), DNA obtained from the immunoprecipitation was analyzed by using qPCR. (F) A portion of the ChIP samples was analyzed by immunoblotting to evaluate AR levels. In a parallel setting, total cell lysates were precipitated with the same AR antibody to evaluate the total cellular AR level in each treatment group.



**Figure 5.**  
Putative mechanisms regulating hsp70-1 expression by AR and AR signaling antagonists.